Inhibition of Caveolin-1 Expression by Incadronate in PC-3 Prostate Cells

KAZUHIRO IGUCHI, SHINJI MATSUNAGA, TORU NAKANO, SHIGEYUKI USUI and KAZUYUKI HIRANO

Laboratory of Pharmaceutics, Gifu Pharmaceutical University, Gifu, Gifu 502-8585, Japan

Abstract. Background: Caveolin-1 is an essential component of caveolae and its expression is known to be increased in human prostate cancer. The reduction of caveolin-1 expression has been reported to decrease the tumorigenic and metastatic potential of prostate cancer. Materials and Methods: Caveolin-1 expression was determined by real-time RT-PCR and Western blot analysis. Results: Incadronate, a third-generation bisphosphonate, was found to inhibit the caveolin-1 mRNA and protein expression in PC-3 prostate cells. The decrease in caveolin-1 mRNA expression by incadronate was prevented by co-incubation with geranylgeranyol, but not with farnesol. Moreover, treatment of GGTTI-286, a geranylgeranyl transferase inhibitor, but not FTI-277, a farnesyl transferase inhibitor, also resulted in the inhibition of caveolin-1 mRNA expression. Conclusion: These results indicate that the decrease in caveolin-1 expression elicited by incadronate is related to the inhibition of protein geranylgeranylation.

Bisphosphonates are analogs of pyrophosphate and are widely used for the treatment of metabolic bone diseases such as osteoporosis, Paget's disease and hypercalcemia with malignant tumor (1-3). Bisphosphonates are also reported to be useful in the prevention of bone disease in patients with advanced cancer (4-8). The mechanisms of the prevention of cancer-related skeletal events such as bone metastasis by bisphosphonates have been investigated in several papers. For example, bisphosphonates had an apoptotic and anti-proliferative effect on osteoclasts and cancer cells, resulting in the inhibition of tumor growth and invasion (9-16). Since prostate cancer often metastasizes to the bone during disease progression, a beneficial effect of bisphosphonates is expected in patients with prostate cancer. Recently, we reported that bisphosphonates inhibited aminopeptidase-N expression, which is known to be involved in the metastasis of prostate cancer (17).

Caveolin-1 is a principal protein of caveolae, specialized plasma membrane microdomains known to play important roles in signal transduction (18). In prostate cancer, caveolin-1 expression increased in most metastatic disease, while reduced expression converted an androgen-insensitive phenotype to an androgen-sensitive one, suggesting that caveolin-1 acts as a metastasis-related and a hormone-resistant associated gene (19-21). Moreover, caveolin-1 was found to function as an anti-apoptotic protein, which activates the protein kinase B (PKB/Akt) signaling pathway leading to cell survival in LNCaP prostate cells (22, 23). A recent study showed a direct relationship between caveolin-1 expression and the development of prostate cancer, indicating caveolin-1 as a potential target for prostate cancer (24, 25).

In the present study, the effect on caveolin-1 expression of incadronate, a third-generation bisphosphonate, was examined, and incadronate was found to inhibit this expression in prostatic PC-3 cells.

Materials and Methods

Materials. Incadronate was kindly supplied by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). Pravastatin and etidronate were purchased from LKT laboratories (St. Paul, MN, USA). Farnesol and geranylgeraniol were from Sigma-Aldrich (St. Louis, MO, USA) and MP Biomedicals (Aurora, OH, USA), respectively. FTI-277 and GGTTI-286 were from Calbiochem (La Jolla, CA, USA). Human PC-3 prostate cells were purchased from the American Type Culture Collection. All other chemicals were of analytical grade.

Cell culture. Human prostatic carcinoma PC-3 cells were cultured at 37°C in RPMI1640 medium containing 10% fetal calf serum, under a humidified atmosphere with 5% CO₂.

RNA isolation and quantitative real-time reverse transcription-PCR (RT-PCR). The PC-3 cells were cultured in 3.5-cm dishes (Nalge Nunc, Rochester, NY, USA) and treated with incadronate. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and first-strand complementary DNA was subsequently synthesized from 5 μg of total RNA using SuperScript III (Invitrogen), as described previously (17). Real-time monitoring of the PCR reactions was performed using the iCycler IQ Real-Time...
PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the iQ SYBR-Green Supermix reagents (Bio-Rad Laboratories). At the end of the PCR, dissociation curve analysis was performed to examine the specificity of the PCR product. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used for the normalization of caveolin-1 mRNA expression. The PCR was performed under the following conditions: 35 cycles of 15 sec at 94°C, 15 sec at 64°C and 30 sec at 72°C for caveolin-1; 35 cycles of 15 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C for GAPDH. The primers used in this study were 5'-GAGCTGAGCGAGAAGCAAGT-3' and 5'-CAATCTTGACCACGTCATCG-3' for caveolin-1; 5'-CAATGACCCTTCATTGACC-3' and 5'-GACAAGCTTCCCGTCTCAG-3' for GAPDH.

Western blot analysis. The PC-3 cells were incubated with 200 μM incadronate for the indicated days. After incubation, the cell surface was washed with ice-cold PBS, pelleted and resuspended in the buffer (20 mM Hepes-NaOH, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 5 mM sodium fluoride, 1% Triton X-100, 10% glycerol, 2 mM DTT, 3 μg/ml leupeptin, 2 μg/ml aprotinin). The cells were lysed by three cycles of freeze and thaw, centrifuged at 12,000 xg for 10 min and the resulting supernatant was used for Western blot analysis. The protein concentrations were quantified using the Bradford assay (26) and 5 μg of each sample was subjected to SDS-PAGE, transferred onto a membrane and probed with anti-caveolin-1 antibody (upper panel). The membrane was also stained with Coomassie brilliant blue to ensure equal loading of the samples (lower panel).

Immunofluorescence. Immunofluorescence was performed as previously described (27). The PC-3 cells were cultured on glass cover slips overnight and were then treated with incadronate for 4 days. After washing with PBS, the cells were fixed in 4% paraformaldehyde for 30 min. The fixed cells were made permeable by treatment with 0.5% Triton X-100 for 5 min at room temperature. The cells were incubated with 2% bovine serum albumin for 30 min at room temperature followed by incubation with anti-caveolin-1 rabbit polyclonal antibody (diluted 1:300; Sigma) for 1 h. After incubation, the cells were rinsed with PBS and stained with TRITC-conjugated, goat anti-rabbit IgG for 1 h in the dark at room temperature.

Statistical analysis. The significance of differences between multiple groups was assessed by one-way analysis of variance followed by the Tukey test.

Results

Inhibition of caveolin-1 expression by incadronate in prostatic PC-3 cells. To investigate the effect of incadronate on caveolin-1 mRNA expression, the PC-3 cells were treated with the indicated concentrations of incadronate for 3 days or with 200 μM incadronate for the indicated days, and the expression level of caveolin-1 was determined by real-time RT-PCR analysis. It can been seen from Figure 1 that the mRNA expression of caveolin-1 decreased in a dose-(A) and time-(B) dependent manner after incadronate treatment. Moreover, Western blot analysis showed that caveolin-1 protein was decreased to 64% and 51% of control at 4 and 6 days after incadronate treatment (as determined by Scion Image software) (Figure 1C). The growth of the PC-3 cells incubated with 200 μM incadronate for 3 days was approximately 60% that of the control cells as determined by alamar blue assay (data not shown; (17)). Only the attached cells were used for the analysis and GAPDH mRNA expression showed similar levels at all doses tested, indicating that the decreased caveolin-1 expression was not due to death by treatment with incadronate.

Inhibition of caveolin-1 expression by incadronate through inhibition of the isoprenoid biosynthesis pathway. Nitrogen-
containing bisphosphonate (and not non-nitrogen-containing bisphosphonate) is known to inhibit the isoprenoid biosynthesis pathway by inhibiting farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase (10, 15, 16). Therefore, to examine whether the isoprenoid biosynthesis pathway was involved in the inhibition of caveolin-1 expression by incadronate, the effects of statin (known to inhibit the formation of isoprenoids) on caveolin-1 mRNA expression were examined. Treatment with pravastatin significantly decreased the expression of caveolin-1 mRNA in PC-3 cells, while treatment with non-nitrogen-containing bisphosphonate (etidronate) did not (Figure 2A). The effects of the intermediates in the isoprenoid biosynthesis pathway (farnesol and geranylgeraniol: cell-permeable forms of farnesyl diphosphate and geranylgeranyl diphosphate, see Figure 4) on caveolin-1 expression in incadronate-treated PC-3 cells were then examined. As shown in Figure 2B, the caveolin-1 mRNA inhibition by incadronate was recovered by co-incubation with geranylgeraniol, but not with farnesol. Treatment with either farnesol or geranylgeraniol alone increased the caveolin-1 mRNA level. Furthermore, the effects of selective inhibitors of farnesyl transferase (FTI-277) and geranylgeranyl transferase (GGTI-286) on caveolin-1 mRNA expression were examined and GGTI-286, but not FTI-277, was found to mimic the effect of incadronate on the caveolin-1 mRNA level (Figure 2C).

Change in the cellular location of caveolin-1 in incadronate-treated PC-3 cells. The distribution of caveolin-1 was examined in incadronate-treated PC-3 cells. As shown in Figure 3, when the cells were exposed to incadronate, caveolin-1 mainly displayed cytoplasmic staining and faint plasma membrane localization, while the caveolin-1 expression of the control PC-3 cells was localized predominantly in the plasma membrane and weakly in the cytoplasm.

Discussion

In the present study, incadronate was found to inhibit the caveolin-1 expression in prostatic PC-3 cells. The inhibitory effect is explained by inhibition of the isoprenoid biosynthesis
Overexpression of caveolin-1 was associated with a severe phenotype in prostate cancer (19) and, recently, the inhibition of caveolin-1 expression in prostatic tumor cells was shown to reduce their tumorigenic and metastatic potential (24), indicating the down-regulation of caveolin-1 expression as a possible target in prostate cancer. The present observation of the inhibition of caveolin-1 expression in the incadronate-treated prostatic PC-3 cells is noteworthy.

Nitrogen-containing bisphosphonates are known to inhibit the key enzyme in the isoprenoid biosynthesis pathway and to interfere with protein prenylation, such as farnesylation and geranylgeranylation of small GTP-binding proteins (10, 15, 16). We, therefore, examined whether the inhibitory mechanism of incadronate on caveolin-1 expression was involved in the inhibition of the isoprenoid biosynthesis pathway, and found the following results: (i) the inhibitory effect of caveolin-1 mRNA expression was also observed in cells treated with pravastatin, a novel inhibitor of the isoprenoid biosynthesis pathway, but not with the non-nitrogen-containing bisphosphonate etidronate (Figure 2A); (ii) the reduction of caveolin-1 expression by incadronate was recovered by co-incubation with the isoprenoid derivative geranylgeraniol, but not with farnesol (Figure 2B); (iii) the geranylgeranyltransferase-I inhibitor (GGTI-286) decreased the expression of caveolin-1 mRNA, whereas the farnesyltransferase inhibitor (FTI-277) showed no significant effect on this expression (Figure 2C). These results strongly suggest that the decrease in caveolin-1 expression elicited by bisphosphonates is involved in inhibition of the isoprenoid biosynthesis pathway, resulting from the reduced geranylgeranyl diphosphate level.

How the inhibition of GGPP formation causes the inhibition of caveolin-1 expression remains unclear. However, several lines of evidence imply a possible explanation. In our previous study, we showed that treatment of PC-3 cells with incadronate led to translocation of the Rap1 small G protein from the membrane (functionally active) to the cytosol fraction (inactive) (17). Since Rap1 membrane association is known to require posttranslational modification of the protein with geranylgeranyl isoprenoid (28), the result indicated that the inhibition of GGPP synthesis by incadronate prevented the membrane transactivation of Rap1. Since Rap1 is known to act as a negative regulator of Ras signaling by trapping Ras effectors in an inactive complex (29), inactivation of Rap1 could activate the Ras/MAPK pathway in incadronate-treated PC-3 cells. Finally, caveolin-1 gene expression was shown to be down-regulated by a Ras/MAPK-dependent signal transduction pathway (30). Taken together, it may be speculated that incadronate-induced Rap1 inactivation leads to Ras activation and then down-regulates caveolin-1 expression. Further studies are required to clarify the mechanism of caveolin-1 down-regulation by incadronate.

The distribution of caveolin-1 was altered from the membrane to cytoplasm during incadronate treatment in the PC-3 cells. Caveolin-1 redistribution was observed in several situations including phosphatase inhibition (31), heat shock (32) and after formation of cell-cell contacts (33). A similar redistribution of endogenous caveolin-1 in incadronate-treated PC-3 cells was observed in okadaic acid-treated A431 cells (31). Although it is unclear whether the altered distribution of caveolin-1 by incadronate is involved in the inhibition of phosphatase activities or not, the change in the distribution is proposed to affect the cellular functions mediated by caveolin-1.

In conclusion, it was found that incadronate induced the inhibition of caveolin-1 expression in prostatic PC-3 cells. Caveolin-1 is expressed in prostate cancer cells at a high level and believed to be relevant to prostate cancer progression. Our findings suggest a possible explanation for the inhibitory effect of the bisphosphonate on tumor growth.

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References


